

# In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13

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The EuroRotaNet surveillance network has conducted rotavirus genotype surveillance since 2007 in 16 European countries. Using epidemiological and microbiological data from 39,786 genotyped rotavirus-positive specimens collected between September 2007 and August 2013, we assessed genotype distribution and age distribution of rotavirus gastroenteritis (RVGE) cases in and out of peak season in 12 countries which were yet to implement routine rotavirus vaccination. In multinomial multivariate logistic regression, adjusting for year, country and age, the odds of infection caused by genotype constellation 2 DS-1-like stains (adjusted multinomial odds ratio (aM-OR) = 1.25; 95% confidence interval (CI): 1.13–1.37;  $p < 0.001$ ), mixed or untypable genotypes (aM-OR = 1.55; 95% CI: 1.40–1.72;  $p < 0.001$ ) and less common genotypes (aM-OR = 2.11; 95% CI: 1.78–2.51;  $p < 0.001$ ) increased out of season relative to G1P[8]. Age varied significantly between seasons; the proportion of RVGE cases younger than 12 months in the United Kingdom increased from 34% in season to 39% out of season (aM-OR = 1.66; 95% CI: 1.20–2.30), and the proportion five years and older increased from 9% in season to 17% out of season (aM-OR = 2.53; 95% CI: 1.67–3.82). This study provides further understanding of the rotavirus ecology before vaccine introduction, which will help interpret epidemiological changes in countries introducing or expanding rotavirus vaccination programmes.

## Introduction

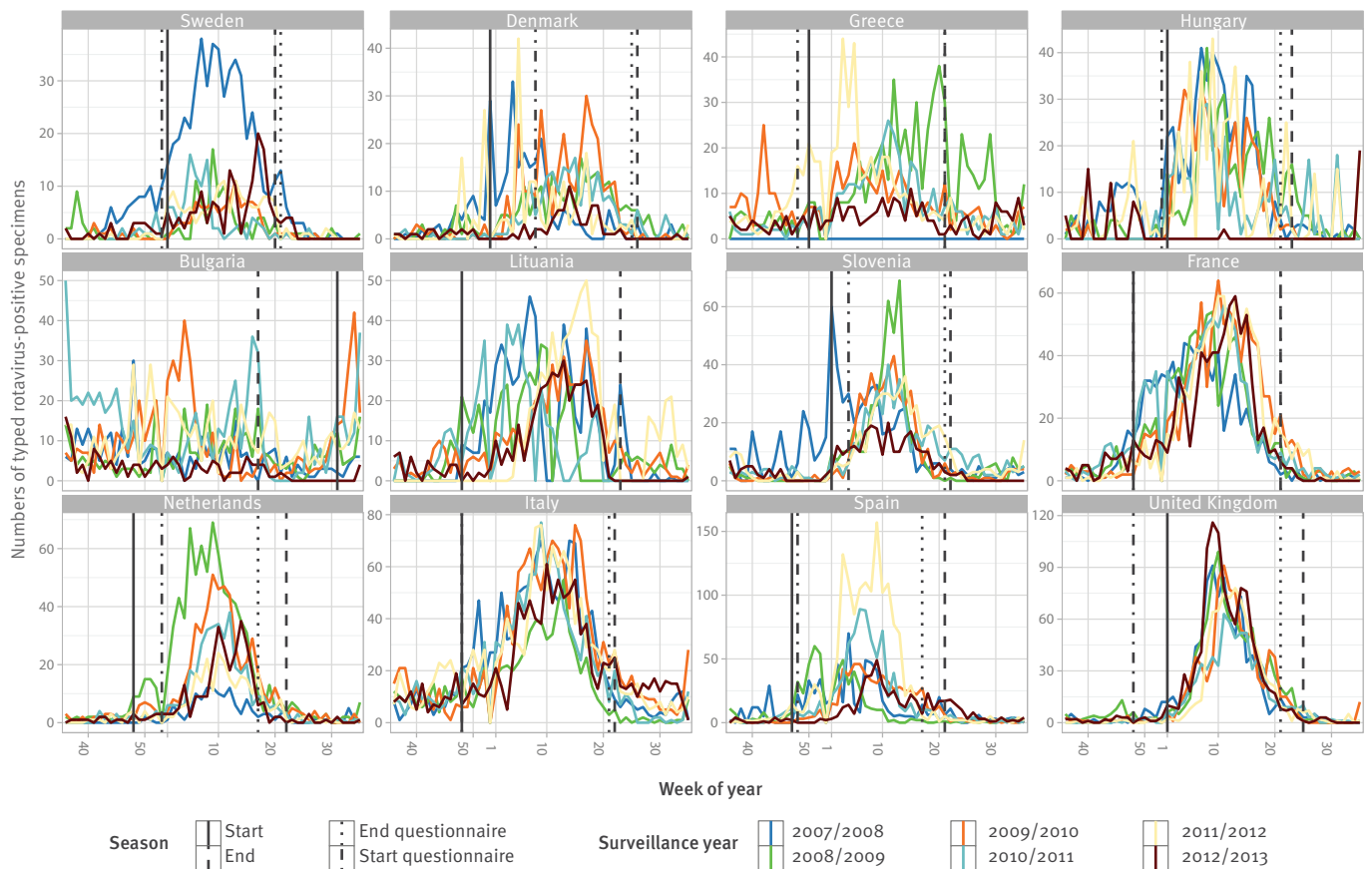
Rotavirus is the most common cause of acute gastroenteritis in children under five years of age, causing an estimated 450,000 deaths per year worldwide, with over 90% of deaths occurring in developing countries [1]. In high-income countries, rotavirus infections result in few deaths but still constitute a substantial healthcare burden and can cause severe morbidity [2,3]. There are eight groups of rotaviruses defined by the middle capsid antigen [4]; the majority of rotavirus gastroenteritis (RVGE) in humans is caused by group A rotaviruses.

Group A rotavirus genotypes are typically further classified into G and P types, based on sequence diversity of the genes encoding the outer viral proteins VP7 (glycoprotein) and VP4 (protease-sensitive protein), respectively [5]. Furthermore, whole genome sequencing has allowed rotavirus strains to be classified into genotype constellations based on a common genomic backbone in which the genotypes of nine of the 11 genes are conserved, while G and P types may vary. Human rotaviruses typically belong to the Wa-like or the DS-1-like genotype constellations [6].

Two oral vaccines, the two-dose monovalent vaccine (Rotarix, GlaxoSmithKline Biologicals, Belgium) and the three-dose pentavalent vaccine (RotaTaq, Merck, United States), have been introduced into a number

**FIGURE 1**

Number of rotavirus specimens typed per week by country and surveillance year, 12 European Union countries, September 2007–August 2013 (n = 39,786)



The season legend shows lines for the start and the end of the rotavirus season as defined by the median threshold method and reported through the questionnaire.

of countries worldwide since their licensure in 2006. Eight European Union countries have included rotavirus vaccines in their routine childhood immunisation schedules and several other countries make the vaccine available through state or private sector health-care [7].

Monitoring the emergence of novel rotavirus genotypes and the potential for genotype replacement and genetic drift is an essential activity of surveillance. This has become more important since the introduction of rotavirus vaccination, as there was some early evidence in countries such as Australia, Brazil and Belgium that vaccination may have contributed to changes in the predominant genotypes, although these changes may also have been the result of natural variation [8,9]. The EuroRotaNet surveillance network, established in 2007 and including 16 countries, has been monitoring rotavirus genotype diversity and year-to-year genotype fluctuations across Europe for eight years [10,11]. Critically, the availability of substantial genotyping and epidemiological data for EuroRotaNet countries provides a baseline for genotype diversity and the epidemiology

of RVGE cases before vaccine introduction. Therefore, while year-to-year differences in genotypes in Europe have been described previously [11,12], this paper reports in-peak season and out-of-season variation of rotavirus genotypes and age of infection for 12 European countries before the introduction of routine vaccination.

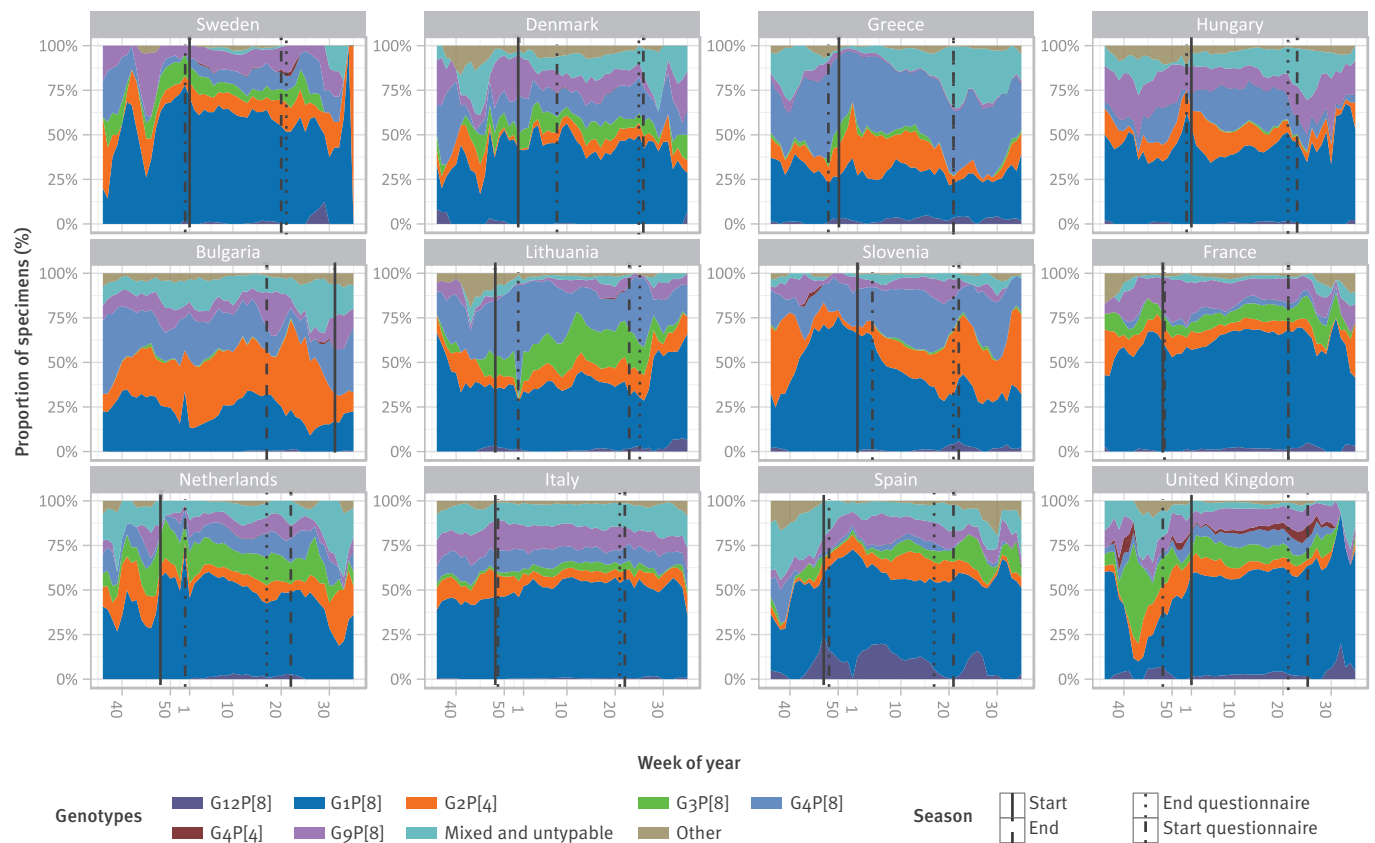
## Methods

### EuroRotaNet

The EuroRotaNet surveillance network was initiated in 2007 and includes 16 countries: Austria, Belgium, Bulgaria, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Lithuania, the Netherlands, Slovenia, Spain, Sweden and the United Kingdom (UK). Data from typed rotavirus-positive faecal specimens is linked to case epidemiological information by participating laboratories and uploaded to a secure web-accessible EuroRotaNet database. The data contained in the EuroRotaNet dataset has been described previously [10,11].

**FIGURE 2**

Number of rotavirus specimens collected in season and out of season, by country, 12 European Union countries, September 2007–August 2013 (n = 39,786)



Case numbers were smoothed using a four-week moving average before conversion to proportions. The season legend shows vertical lines for the start and end of the rotavirus season, defined, respectively, by the median threshold method and through the questionnaire.

### Study area

Twelve countries from EuroRotaNet were included in the study. Data from Austria, Belgium, Finland and Germany were excluded from the analysis because rotavirus vaccination was either routine or widespread (>35%) in these countries during the study period [13].

### Samples

Study samples included rotavirus-positive faecal samples from mostly sporadic gastroenteritis cases; if associated with outbreaks, only a single sample per outbreak was submitted for routine diagnostic testing at sentinel participating EuroRotaNet laboratories and typed using standardised G and P typing methods [12,14]. Diagnostic testing protocols varied between countries [12,14].

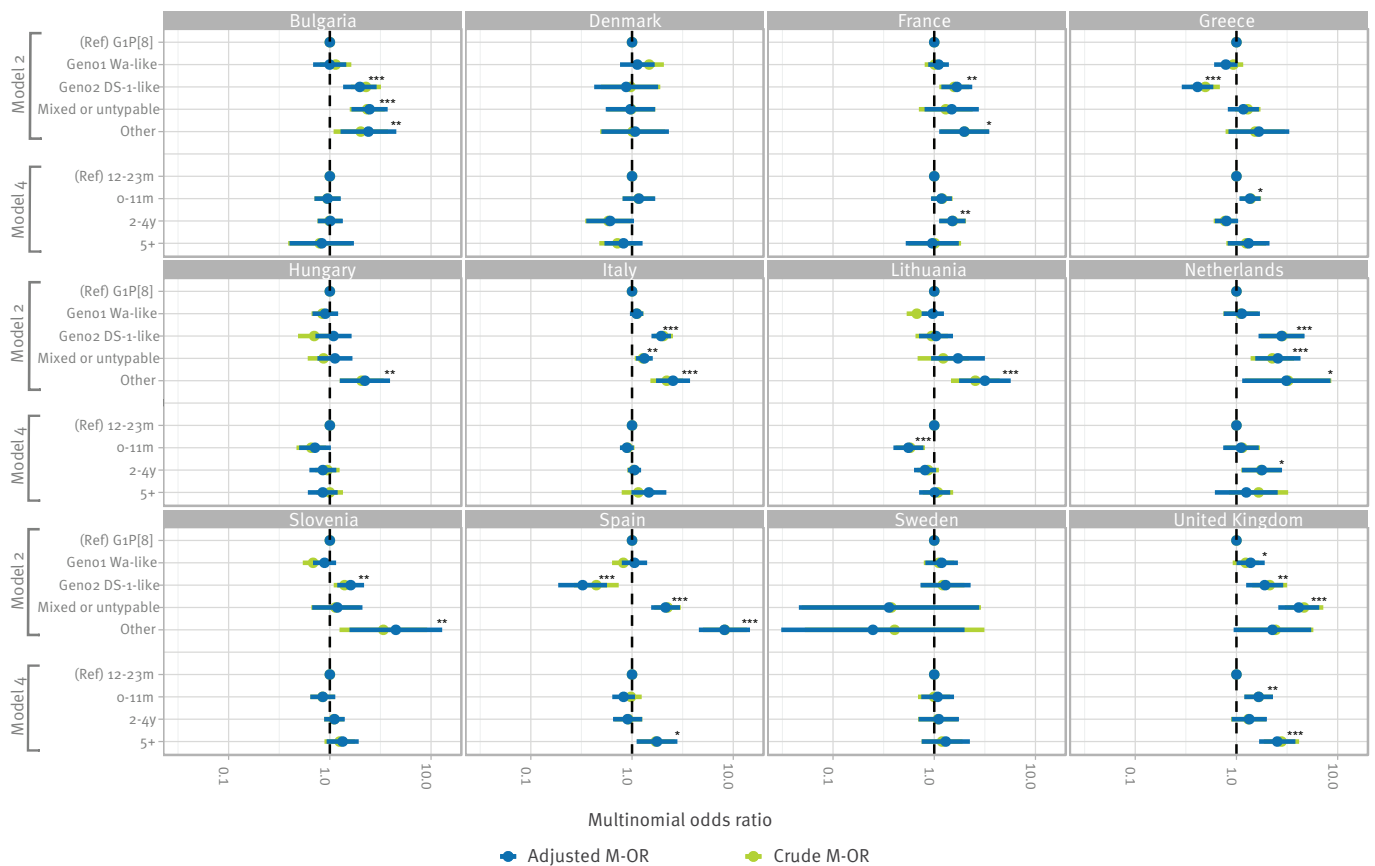
### Data and survey

Details on case age, sex and country, specimen collection date and rotavirus genotyping results for a total of six rotavirus seasons spanning September 2007 to August 2013 were included in this study. Greece joined EuroRotaNet in 2008; therefore, for Greece only five rotavirus seasons were included in the analysis, spanning September 2008 to August 2013.

Data for each of the 12 countries were pooled for the study period. Age groups of cases (0–11 months, 12–23 months, 2–4 years and ≥5 years) were constructed using date of birth and date of specimen collection. Genotypes were categorised as ‘G1P[8]’, ‘genotype-constellation 1 (Wa-like: G3P[8], G4P[8], G9P[8] and G12 P[8])’, ‘genotype-constellation 2 (DS-1-like: G2P[4] and G8P[4])’, ‘mixed and untypable’, and less common genotypes were combined under the category ‘other’. Although G1P[8] is considered part of genotype-constellation 1 (Wa-like), we grouped it separately because of its high prevalence across Europe [12]. A derived binary variable was constructed to denote weeks within a country’s peak season and non-peak rotavirus seasons, and was developed by pooling country-specific weekly specimen frequencies over the study period to calculate the overall median weekly specimen frequency. We used the country-specific median value as a threshold for defining the start and end of the peak rotavirus season over the study period. Consequently, a weekly specimen frequency greater than or equal to the median identified weeks as in-season and a weekly frequency less than the median identified weeks as out-of-season. A consecutive period of two weeks

**FIGURE 3**

Country-stratified crude and adjusted multinomial odds ratios for genotypes occurring out of season vs in season (model 2; n = 39,786) and for age group (model 4; n = 39,007), 12 European Union countries, September 2007–August 2013



Multinomial odds ratios (M-OR) for out-of-season occurrence were estimated with multinomial logistic regression with the outcome variable genotype (G1P[8] as the reference group) (model 2) or age group (12–23-month-olds as the reference group) (model 4). Models were additionally adjusted for surveillance year (September–August) and either age group or genotype.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Blue line: adjusted M-OR; green line: crude M-OR; Geno1 Wa-like: genotype-constellation 1 Wa-like; Geno2 DS-1-like: genotype-constellation 1 DS-1-like.

above or below the threshold was required to identify the beginning and end of a season to ensure season identification was robust to stochastic fluctuations.

To identify additional detail on country-specific in-season and out-of-season testing practices, we constructed a brief semi-structured questionnaire using SelectSurvey.Net [15]. The questionnaire was distributed to EuroRotaNet collaborators by email in July 2014. The questionnaire included questions on duration of rotavirus season within the country, typical diagnostic testing practices, identification of changes to testing practices during the study period (including dates of any changes), positivity rate and proportion of positive samples typed. The questionnaire also asked for details on any age restrictions to testing or other algorithms that may have influenced testing and whether these may have changed between rotavirus seasons.

## Statistical analysis

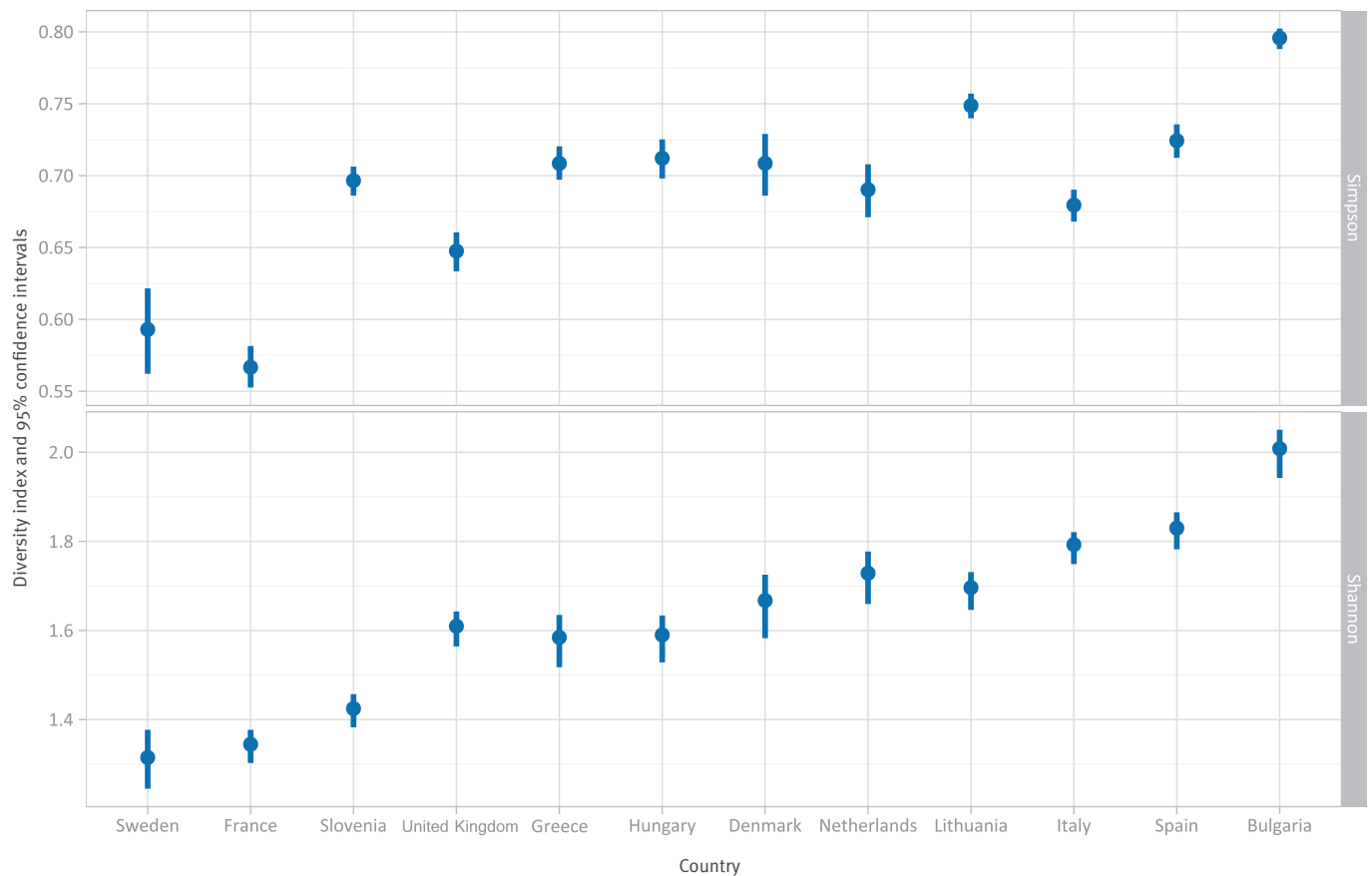
### Models relating genotypes and age of cases to season

To investigate differences in circulating genotypes and age of cases out of season vs in season, we fitted a series of mixed-effect multinomial logistic regression models with the two main outcomes: genotype group and age group of cases. Model fitting was based on variables identified a priori and used categorical variables for genotype group (reference group: G1P[8]), age group of the case (reference group: 12–23-month-olds), surveillance year (September to August) and country, and the binary season indicator was the covariate term of interest. The following adjusted models were then fit:

**Genotype full model (model 1):** genotype as the outcome variable; season, age group of case and surveillance year as covariates; and a random intercept for country.

**FIGURE 4**

Rotavirus genotype diversity measured using Shannon's index and Simpson's index of diversity, with 95% confidence intervals, by country, 12 European Union countries, pooled September 2007–August 2013 (n = 39,786)



**Genotype country-stratified model (model 2):** model 1 but without a random intercept for country; effectively a series of country-specific multinomial logistic regressions.

**Age group full model (model 3):** age group of cases as the outcome variable; season, genotype and surveillance year as covariates; and a random intercept for country.

**Age group country-stratified model (model 4):** model 3 but without a random intercept for country; effectively a series of country-specific multinomial logistic regressions.

Each model was initially run as a univariate analysis including only the binary season indicator as the covariate term of interest. Multinomial odds ratios (M-OR; also referred to as RR ratios), 95% confidence intervals (CI) and the associated p values for season were calculated from the Wald test. Results were considered significant at  $p < 0.05$ . In supplementary analyses, mixed-effects multinomial logistic regression investigated the relationship between age group and genotype group regardless of season, therefore model 1 was re-run excluding season as a covariate (model 5).

### Strain diversity

Rotavirus genotype diversity in the 12 European countries studied was compared using two established biodiversity indices, Simpson's index of diversity and Shannon's index [16]. Simpson's index of diversity (D) represents the probability that two randomly chosen rotavirus genotypes will have different G and P types and is calculated as  $1 - \lambda$ , where  $\lambda = \sum(p_i^2)$  and  $p_i$  is the proportional abundance of a genotype  $i$ . Shannon's index ( $H'$ ) quantifies the uncertainty in predicting the rotavirus genotype identity of an individual sample that is taken at random from the dataset and is calculated as  $H' = -\sum(p_i \times \ln(p_i))$ . Confidence intervals were estimated using bootstrap resampling methodology and differences in season and out of season were compared for each country.

### United Kingdom representativeness test

Linear regression was used to assess the representativeness of the seasonality of genotyped rotavirus data in comparison to all confirmed laboratory reports of rotavirus infection in the UK. The regression takes the form,  $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$ , where  $Y$  is the number of confirmed laboratory reports of rotavirus infection,  $X$  represents the covariates (number of genotyped rotavirus specimens and month of specimen),  $\alpha$  is the intercept term and  $\varepsilon$  represents the error term.

**TABLE 1**

Number of rotavirus specimens collected in season and out of season, by country, 12 European Union countries, September 2007–August 2013 (n = 39,786)

Country	Total specimens	In season		Out of season		In-season weeks (calendar weeks)
	Number	Number	%	Number	%	Number
Bulgaria	2,627	2,296	87	331	13	31–17
Denmark	1,392	1,192	86	200	14	1–26
France	5,044	4,584	91	460	9	48–21
Greece <sup>a</sup>	2,115	1,447	68	668	32	50–21
Hungary	2,263	1,835	81	428	19	1–23
Italy	6,955	5,685	82	1,270	18	48–22
Lithuania	2,990	2,582	86	408	14	49–23
The Netherlands	2,508	2,346	94	162	6	48–22
Slovenia	2,779	2,272	82	507	18	1–22
Spain	4,609	4,227	92	382	8	47–21
Sweden	1,232	1,030	84	202	16	1–20
United Kingdom	5,272	5,014	95	258	5	1–25
<b>Total</b>	<b>39,786</b>	<b>34,510</b>	<b>87</b>	<b>5,276</b>	<b>13</b>	<b>NA</b>

NA: not applicable.

<sup>a</sup> Data between September 2008 and August 2013.

All data handling and statistical analyses were performed using R Version 3.1.2. and Stata Version 14 [17,18]. The R packages ‘Vegan’ and ‘boot’ were used for analysis of genotype biodiversity [19,20]. Data tables are available through the EuroRotaNet website or available on request from the authors [10].

## Results

### Descriptive statistics

A total of 39,786 rotavirus-positive specimens from 12 countries were typed between September 2007 and August 2013. Rotavirus seasonality for genotyped rotavirus-positive specimens was variable across the countries studied (Figure 1). In the UK, the peak of the rotavirus season was well defined every year, typically occurring between weeks 10 and 12. The representativeness test for the UK confirmed that the seasonal pattern of the typed rotavirus specimens was representative of laboratory-confirmed rotavirus cases in the UK (adjusted  $R^2=0.75$ ). Table 1 shows the total number of typed specimens for each country, the number in season and out of season, and the number of weeks per year classified as in season. The proportion of specimens referred for typing that were collected in season ranged from 68% in Greece to 95% in the UK.

The predominant genotype overall was G1P[8], representing 48% of the specimens included in the analysis (range: 24% in Bulgaria to 63% in France). G1P[8] predominated in all countries except Greece and Bulgaria where the predominant genotypes were G4P[8] and G2P[4], respectively (Table 2). Children younger than five years contributed 93% of the specimens (range: 77% in Denmark to 97% in Bulgaria, France and Italy).

It is difficult to distinguish aberrant events due to the data’s stochastic nature (Figure 1). However, some can be explained by outbreaks of particular genotypes. For instance in Spain, the increased incidence during the 2011/12 surveillance year was due to an outbreak of G12P[8] in the north-eastern province of Gipuzkoa.

### Genotypes in season and between rotavirus seasons

Across all countries studied, when adjusting for country, surveillance year and age group, the adjusted multinomial odds ratio (aM-OR) of infection caused by strains with DS-1-like genotype-constellation (aM-OR = 1.25; 95% CI: 1.13–1.37;  $p<0.001$ ), mixed or untypable genotypes (aM-OR = 1.55; 95% CI: 1.40–1.72;  $p<0.001$ ) and less common genotypes (group: ‘other’; aM-OR = 2.11; 95% CI: 1.78–2.51;  $p<0.001$ ) increased out of season relative to G1P[8], while infection caused by strains with Wa-like genotype constellation declined (aM-OR = 0.93; 95% CI: 0.86–1.00;  $p=0.04$ ) (model 1).

In country-stratified analyses (model 2), the proportional distribution of rotavirus genotypes varied by country (Figure 2). There were significant differences in the proportional representation of genotypes from specimens collected in season and out of season in 10 of the 12 countries studied. In these 10 countries, out-of-season specimens were more likely to belong to a less common genotype (group: ‘other’) than specimens collected in season (Figure 3). However, this was only significant in eight countries, with the highest aM-OR observed in Spain (aM-OR = 8.18; 95% CI: 4.57–14.64) and Slovenia (aM-OR = 4.49; 95% CI: 1.56–12.88). DS-1-like genotypes were significantly more likely to occur out of season in

**TABLE 2**

Distribution of rotavirus genotypes and age of infection in 12 European Union countries, September 2007–August 2013 (n = 39,786)

Genotype	Bulgaria n = 2,627		Denmark n = 1,392		France n = 5,044		Greece <sup>a</sup> n = 2,115		Hungary n = 2,263		Italy n = 6,955		Lithuania n = 2,990		The Netherlands n = 2,508		Slovenia n = 2,779		Spain n = 4,609		Sweden n = 1,232		United Kingdom n = 5,272		Total n = 39,786			
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%		
G1P[8]	636	24	614	44	3,182	63	566	27	934	41	3,636	52	1,140	38	1,247	50	1,195	43	2,120	46	747	61	2,958	56	18,975	48		
G2P[4]	682	26	87	6	339	7	310	15	273	12	471	7	276	9	186	7	525	19	461	10	119	10	314	6	4,043	10		
G4P[8]	605	23	198	14	154	3	768	36	414	18	632	9	742	25	242	10	729	26	133	3	110	9	403	8	5,130	13		
G9P[8]	267	10	159	11	686	14	37	2	288	13	878	13	138	5	189	8	181	7	564	12	132	11	565	11	4,084	10		
G3P[8]	12	0	115	8	392	8	47	2	13	1	274	4	516	17	339	14	23	1	239	5	83	7	511	10	2,564	6		
G12P[8]	4	0	3	0	79	2	51	2	14	1	36	1	30	1	44	2	27	1	589	13	8	1	145	3	1,030	3		
G8P[4]	1	0	2	0	1	0	0	0	0	0	1	0	3	0	4	0	1	0	4	0	4	0	122	2	143	0		
Mixed and untypable	325	12	144	10	110	2	299	14	251	11	887	13	82	3	223	9	82	3	429	9	15	1	186	4	3,033	8		
Other	95	4	70	5	101	2	37	2	76	3	140	2	63	2	34	1	16	1	70	2	14	1	68	1	784	2		
Age group <sup>b</sup>	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
0–11 months	754	29	352	25	2,616	53	837	40	440	20	2,208	32	569	19	1,145	46	554	20	1,841	42	413	34	1,698	35	13,427	34		
12–23 months	879	34	520	37	1,402	28	511	24	552	25	2,124	31	893	30	752	30	1,089	39	1,579	36	443	37	1,818	37	12,562	32		
2–4 years	892	34	195	14	760	15	665	31	731	33	2,421	35	1,205	40	457	18	893	32	787	18	192	16	918	19	10,116	26		
5–14 years	76	3	39	3	113	2	99	5	365	16	164	2	309	10	46	2	122	4	135	3	15	1	164	3	1,647	4		
15–64 years	8	0	143	10	28	1	1	0	91	4	12	0	13	0	33	1	82	3	60	1	62	5	107	2	640	2		
≥ 65 years	1	0	143	10	26	1	0	0	38	2	3	0	0	0	62	2	35	1	22	1	90	7	195	4	615	2		

<sup>a</sup> Data between September 2008 and August 2013.

<sup>b</sup> There were 779 specimens with unknown case age. Percentages are calculated from total specimens with known case age.

Bulgaria (aM-OR = 1.98; 95% CI: 1.35–2.90), France (aM-OR = 1.67; 95% CI: 1.18–2.37), Italy (aM-OR = 1.94; 95% CI: 1.56–2.42), the Netherlands (aM-OR = 2.79; 95% CI: 1.65–4.71), Slovenia (aM-OR = 1.61; 95% CI: 1.18–2.18) and the UK (aM-OR = 1.90; 99CIs: 1.25–2.90), whereas they were less likely to occur out of season in Spain (aM-OR = 0.32; 95% CI: 0.19–0.57) and Greece (aM-OR = 0.41; 95% CI: 0.29–0.59). Untypable and mixed genotypes had significantly higher proportional representation out of season in Bulgaria (aM-OR = 2.47; 95% CI: 1.63–3.73), Italy (aM-OR = 1.32; 95% CI: 1.10–1.60), the Netherlands (aM-OR = 2.57; 95% CI: 1.53–4.29), Spain (aM-OR = 2.14; 95% CI: 1.55–2.98) and the UK (aM-OR = 4.13; 95% CI: 2.59–6.57). Only the UK (aM-OR = 1.38; 95% CI: 1.00–1.90) showed a significant change in the proportional representation of other genotype-constellation 1 (Wa-like) genotypes out of season compared with in season. Although Sweden and Denmark were the only two countries that did not show significant changes in genotype distribution out of season compared with in season, they had very different genotype distributions (Table 2).

### Age of cases in season and out of season

Across all countries studied, when adjusting for country, surveillance year and genotype, the aM-OR of infection in two- to four-year-olds (aM-OR = 1.13; 95% CI: 1.04–1.22;  $p < 0.01$ ) and in those five years and older (aM-OR = 1.13; 95% CI: 1.00–1.27;  $p = 0.04$ ) increased out of season relative to the younger children 12–23 months of age, while declining in those younger than 12 months (aM-OR = 0.92; 95% CI: 0.85–0.99;  $p = 0.03$ ) (model 3). Country-stratified analyses (model 4) showed that when adjusting for genotype and surveillance year, half of the countries experienced significant variation in the age group of cases out of season as compared with in season (Figure 3). Cases five years and older constituted a higher proportion of the out-of-season than of the in-season cases in Greece, Italy, the Netherlands, Slovenia, Spain, Sweden and the UK. This difference was only significant in Spain (aM-OR = 1.76; 95% CI: 1.11–2.81) and the UK (aM-OR = 2.53; 95% CI: 1.67–3.82). In France (aM-OR = 1.51; 95% CI: 1.12–2.04) and the Netherlands (aM-OR = 1.79; 95% CI: 1.13–2.82), two- to four-year olds were significantly more commonly represented out of season compared with in season. Lithuania had significantly smaller proportions of cases 0–11 months of age (aM-OR = 0.56; 95% CI: 0.39–0.78) in season compared with out of season, whereas Greece (aM-OR = 1.36; 95% CI: 1.07–1.73) and the UK (aM-OR = 1.66; 95% CI: 1.20–2.30) had a significantly higher proportion of cases younger than 12 months out of season compared with in season.

### Relationship between age of cases and genotype group

There was a significant association between increasing age and the genotypes causing disease regardless of season. Those five years and older were more likely to be infected with non-G1P[8] genotypes than those younger than five years (model 5). This was most

pronounced in the DS-1-like genotype-constellation (aM-OR = 2.56; 95% CI: 2.27–2.90;  $p < 0.001$ ), but also significant for mixed or untypable genotypes (aM-OR = 1.92; 95% CI: 1.65–2.23;  $p < 0.001$ ), less common genotypes (group: 'other') (aM-OR 2.32; 95% CI: 1.79–3.02;  $p < 0.001$ ) and Wa-like genotype constellations (aM-OR = 1.15; 95% CI: 1.04–1.27;  $p < 0.01$ ). The 0–11-months-old infants were also more likely than the reference group (12–23-month-olds) to be infected with mixed or untypable genotypes (aM-OR = 1.23; 95% CI: 1.11–1.35;  $p < 0.001$ ) and less common genotypes (group: 'other') (aM-OR = 1.30; 95% CI: 1.08–1.56;  $p < 0.01$ )

### Genotype diversity

Sweden and France had the lowest genotype diversity and Bulgaria the highest (Figure 4). Age group analysis showed that genotype diversity was highest in the age group five years and older in six of 12 countries based on Shannon's index and in eight of 12 countries based on Simpson's index of diversity. When cases five years and older were compared with the reference category of 12–23-month-olds, diversity was significantly higher in Shannon's index, Simpson's index of diversity or both indices in Denmark ( $H'$ :  $p = 0.021/D$ :  $p = 0.585$ ), Italy ( $H'$ :  $p < 0.001/D$ :  $p < 0.001$ ), the Netherlands ( $H'$ :  $p = 0.192/D$ :  $p = 0.003$ ), Sweden ( $H'$ :  $p < 0.001/D$ :  $p < 0.001$ ) and the UK ( $H'$ :  $p < 0.001/D$ :  $p < 0.001$ ). When comparing genotype diversity in season with out-of-season genotype diversity, only Italy and the UK showed significant differences in genotype diversity. Both Shannon's index and Simpson's index of diversity showed significantly higher genotype diversity out of season in Italy ( $H'$ :  $p = 0.012/D$ :  $p < 0.001$ ) whereas only Simpson's index of diversity indicated significantly higher genotype diversity out of season in the UK ( $H'$ :  $p = 0.098/D$ :  $p = 0.003$ ).

### Survey

All countries responded to the survey. Only Hungary indicated that they had reporting laboratories which did not test for rotavirus all year round. There was little variation in the temporal definition of the peak rotavirus season between the questionnaire responses and the statistical coding specified in the Methods chapter. The exceptions were Bulgaria and Denmark. The questionnaire response for Bulgaria specified no seasonality, whereas we identified weeks 31 to 17 for this analysis. For Denmark, the questionnaire response specified peak rotavirus season as March to June, while for the analysis, we defined it as weeks 1 to 26 (i.e. beginning in January).

Diagnostic tests used included enzyme-linked immunosorbent assay (ELISA), dual adenovirus/rotavirus rapid immunochromatographic tests (RIT), real-time RT-PCR, single rotavirus RIT, and electron microscopy. Dual RIT (9/12 responses) and ELISA (8/12 responses) were the most common tests. During the time period studied, it was reported that one laboratory in France had changed testing from latex agglutination to Dual RIT,



and laboratories in four other countries had changed from ELISA to real-time RT-PCR or increased its use.

Age testing policies were variable across countries. Italy, Spain and the UK specified that they routinely test only children younger than five years, while other countries either included older children or tested all ages. Only one laboratory in France was identified as changing age group testing policies out of season. This laboratory specified that it changed from testing all ages to testing immunocompromised cases and children younger than five years only. In addition, a variety of factors were reported as influencing decision to test, but clinician request was selected in every survey response. Other common factors influencing decision to test included nosocomial outbreaks of acute gastroenteritis in a paediatric ward (10/12 responses) and diarrhoeal outbreaks in a nursery (8/12 responses). Apart from the aforementioned laboratory in France, respondents indicated that factors influencing testing for rotavirus were the same in season as out of season, and all countries stated that their decision to genotype did not vary in season and out of season.

## Discussion

Significant differences in the circulating rotavirus genotypes in season compared with out of season were observed across the countries studied. Genotype G1P[8] was dominant in season but this dominance declined out of season in most countries, whereas the proportion of other less abundant genotypes increased out of season. Other than the dominance of G1P[8] in most countries, there was little consistency in genotype distribution across countries studied, highlighted by the country-to-country variation in genotype diversity and relative genotype dominance. For instance in Bulgaria, no genotype was identified as dominant, and the survey results further elucidated that Bulgaria does not appear to have a well-defined rotavirus season.

The analysis also showed that there were clear seasonal differences in the age distribution among rotavirus cases out of season vs in season. These differences were not consistent across all the countries studied. Generally, the proportion of cases five years and older increased out of season and in most countries, genotypes found in cases aged five years and older were more diverse than genotypes identified among younger age groups regardless of season. Relative to younger cases, cases aged at least five years were more likely to be infected with a non-G1P[8] genotype, in particular genotypes from the DS-1-like genotype constellation.

The relative decline of G1P[8] genotypes out of season is common in European countries and by definition coincides with a flattening of incidence, similar to countries with smoother incidence throughout the year, such as Bulgaria, where no single genotype is dominant. This pattern is also reflected in observations from countries which have introduced rotavirus vaccination, reinforcing the importance of understanding

the pre-vaccine ecology of rotavirus infection across Europe for interpreting changes in rotavirus genotype distribution, seasonality and age of infection after vaccine introduction [21–24].

Seasonal and age group differences in the distribution of rotavirus genotypes may be driven by differential virus fitness among susceptible and partially immune hosts. Younger children, who are more susceptible, may be preferentially infected by the G1P[8] genotype, which given its predominance in most countries may be better adapted to the host or to transmission. The out-of-season decline in G1P[8] dominance may then be driven by the accumulation of homotypic immunity to G1P[8] in the community during the rotavirus season, reducing the number of susceptible hosts out of season and enabling the potentially less fit non-G1P[8] genotypes to infect those who have homotypic immunity from previous exposure to G1P[8] (24–60-month-olds may only have partial protection due to limited number of exposures) and older individuals infected with other genotypes to which cross-protection may be incomplete [25,26]. Indeed, a Mexican study showed that natural rotavirus infection reduces host susceptibility after each infection and that secondary infections are more likely to be caused by a different genotype than the one causing the first infection [25]. Furthermore, this explanation may be consistent with previous findings in which birth cohort effects were identified as potential drivers for differences in seasonality across the United States (US) [27].

Such differences between heterotypic and homotypic protection conferred by the dominant G1P[8] genotype support results from vaccine efficacy and observational studies of the monovalent Rotarix vaccine, which show that although the vaccine does protect against completely heterologous genotypes (e.g. G2P[4]), it may do so to a lesser extent [28–31].

The analysis also showed that mixed and untypable genotypes proportionally increased in a number of countries out of season. The types available for partially typed rotaviruses (G or P type unobtainable) appear to be representative of the more commonly found types (typically G1 or P[8]). Insufficient sensitivity of the typing procedures is the most likely cause for the typing failures [32]. These samples may, therefore, contain lower viral loads, which are likely to be associated with infections in previously exposed individuals with partial protection and/or subclinical infections.

Therefore, a plausible explanation for the increase in the proportional representation of older children and adults and of mixed and untypable genotypes out of season might be the accidental detection of an (asymptomatic) rotavirus infection in previously exposed individuals protected from severe RVGE, coinciding with infection by another pathogen causing gastrointestinal symptoms that has peak incidence in the summer months, such as some gastrointestinal bacterial

pathogens. This could be supported by a study in the US that found that in adults admitted to hospital with diarrhoea, rotavirus was as commonly detected as bacterial gastrointestinal pathogens [33]. Furthermore, pre-vaccine studies suggest that there are high symptomatic and asymptomatic infection rates in adults regardless of epidemic season and that re-infection in adults persists across the year, which may suggest that older children and adults may be a reservoir from which the winter/spring paediatric epidemic emerges [34–36].

Our findings also suggest rapid genotype cycling from in-season to out-of-season periods and, as noted by Pitzer et al. [26], this could be caused by relatively stronger homotypic immunity than heterotypic immunity in the population, which renders the less common genotypes increased fitness, permitting them to persist in the population [26,37]. Moreover, age increases among RVGE cases as the predominant genotype declines, and the rapid cycling to less common genotypes out of season may explain the proportional increase in two- to four-year-olds and those five years and older seen in our analysis out of season [26,27]. However, an increase in those five years and older out of season may also be influenced by delayed transmission to this group because of mixing and contact patterns in younger children and infants. Additionally, the change to older age groups and less common genotypes out of season could potentially be related to importations associated with travel.

Interpretation of the proportional increase in specimens from those five years and older is, however, complicated by testing practices. The survey suggests that laboratories in some countries routinely test for rotavirus only in children younger than five years or, in some cases, those younger than 18 years, while limited testing occurs in older age groups. However, only one laboratory among the study countries reported changes in either age-specific testing procedures or clinician requests in season compared with out of season. Also, specifically in the UK, published guidance suggests a consistent testing algorithm all year, indicating that the reported variation in age of infection is representative [38].

Unfortunately, there is no apparent explanation for increases in the proportion of rotavirus-positive infants younger than 12 months out of season in Greece and the UK and for the decline in Lithuania. Findings are unlikely to be explained by seasonal birth rates as birth rate seasonality is similar in all the countries studied, suggesting that other factors, such as low heterotypic immunity conferred by previous infection, may be responsible [39].

We have described a number of potential hypotheses that may contribute to the observed differences in genotype and age distribution in season and out of

season. However, we recognise this is not exhaustive and there may be other plausible hypotheses.

### Strengths and limitations

Our analysis benefited from using an established surveillance system that has achieved consistency over a number of years. We supplemented our understanding of these data with a network-wide survey of testing practices. Nevertheless, there are limitations. Firstly, the sample size of rotavirus-positive samples typed was calculated based on detecting genotypes with a prevalence of at least 1% and, depending upon the country population size and estimated rates of rotavirus infection, are therefore not representative of the incidence of RVGE [11]. Secondly, it is unknown how many samples are referred for rotavirus diagnosis or how many are positive in routine diagnostic laboratories given that rotavirus is not a notifiable disease in many of the countries studied. For this reason we were unable to provide the proportion of positive samples each country submits for genotyping. Consequently we could not quantify the effect of sampling bias on out-of-season increases in less common genotypes, and the smaller number of cases out of season means that we must be aware of random variation when considering the findings. However, the study design helped to increase precision by pooling data over a number of seasons. Thirdly, data completeness of sex in the EuroRotaNet database was inconsistent across the countries studied. Previous analysis of EuroRotaNet data has shown no differences in genotype distribution between the sexes [11]. For these reasons sex was excluded from our models. Fourthly, the survey has shown that diagnostic procedures can vary slightly between countries and that a small number of laboratories have changed testing practices during the study period, which may have influenced the number of detected cases. However, a study in the UK found no association between number of laboratory reports and proportion of cases diagnosed by each diagnostic method [40]. Fifthly, even though countries included in the study had either low vaccination coverage (<35%) or total absence of routine rotavirus vaccination [13], we have been unable to account for the effect of low-level vaccination in countries in which vaccine is available in regions and/or in the private health care sector, or the effect of routine vaccination in neighbouring countries on our findings. Finally, it is important to acknowledge that EuroRotaNet data are likely to be representative only of moderate to severe cases because in many countries, rotavirus is not a notifiable disease and because symptoms often resolve without healthcare contact.

### Conclusions

This study shows that rotavirus genotype distribution in Europe is variable and that most countries included in this study experience variation in genotypes typed from specimens collected during the peak rotavirus season compared with the out-of-season periods. Changes in age of infection between peak rotavirus season and out-of-peak season may be due to lower

cross-protection against heterotypic genotypes. These findings raise several questions about the genotype reservoirs and genotype persistence that may help direct future research to understand the temporal variability in the environment and in hosts. In addition, the true burden and epidemiology of rotavirus infections in adults and older children are not well understood due to age-exclusive testing policies, but the study further indicates that this could be critical to understanding re-infection and transmission that persists to re-ignite the epidemic season each year.

Finally, of the countries studied here, the UK has since introduced rotavirus vaccination into the childhood immunisation schedule. Critically, this work provides important pre-vaccine ecological data for the UK and other European countries introducing or expanding rotavirus vaccination programmes.

### Members of the EuroRotaNet

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### Conflict of interest

Rotarix is developed and licensed by GSK Biologicals. NC has received research grant support from GSK Biologicals and honoraria for participation in GSK Rotavirus Vaccine Advisory Board Meetings.

### Authors' contributions

DH participated in study design, developed the survey, performed data management, conducted the analysis and wrote the manuscript. EuroRotaNet members contributed to study design and data collection. RV contributed to study design and survey design. JMR contributed to the analysis. VEP contributed to the analysis. NF participated in study design and contributed to the analysis. NC contributed to interpretation of data. MIG conceived of the study; contributed to survey design and data collection. All authors contributed to the interpretation of the data, drafting the article, and final approval of the version to be published. No person or persons other than the authors listed have contributed significantly to the study or manuscript preparation.

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